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13. ABSTRACT (Maximum 200 Words)

This report describes the ongoing project to fillout shell space on the third floor and basement of Othmer Hall, the new home for the University of Nebraska-Lincoln Biological Process Development Facility. At the detailed design is completed and construction is nearly half completed with the expected completion date being December 2003. The third floor will house all of the process research capabilities, i.e. molecular biology, fermentation, cell culture, analytical methods, quality control, purification development and media prep.

This report also describes research on the expression of antibodies in Chinese Hamster Ovary (CHO) cells against serotype A botulinum neurotoxin. We have been able to clone and successful express the full intact S-25 antibody provided by Dr. Jim Marks of UCSF at 30 mg/L in 250 mL spinner shake flasks. This product has been purified and sent to Dr. Leonard Smith of USAMRIID for evaluation. Results are pending.

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Introduction

The funding for this project is comprised of two components. The first component, which represents 95% of the funding, is dedicated to filling out shell space on the third floor and basement of Othmer Hall, the new home for the Biological Process Development Facility. The other 5% is dedicated to research focused on the expression of antibodies against the botulinum neurotoxins in Chinese Hamster Ovary (CHO) cells. This report will provide a summary of the past year's activity on the design and initiation of construction. With regards to the antibody project, activity was significantly delayed due to the inability of another USAMRMC contractor to deliver a humanized antibody gene that UNL required to transform the CHO cells. UNL needed to improvise on laboratory space to begin the antibody project since construction project includes the building of new cell culture laboratory space that is currently not available at UNL. UNL was able to make significant progress on the BoNT antibody project and the work summarized below is through September 1, 2003.

Body

Construction Project

The majority of the first year was spent designing the third floor and basement facility, which are currently unfinished shell space. This was performed through a local architectural firm, Davis Design, and required 9 months to complete. The result of this phase of the project was a set of bid documents. Through a competitive process the contract was awarded to Builder, Inc of Lincoln Nebraska and construction was initiated in March 2003 with completion expected in December 2003.

The third floor of Othmer Hall will house research laboratory and administrative and staff office space, while the basement is designed for research pilot plants. A description of the space is provided.

The third floor will accommodate all of the research capabilities of the BPDF, along with a Class 10,000 laboratory dedicated to production of Master Cell Banks (MCB) and Working Cell Banks (WCB) and three bioengineering research laboratories for two faculty members in the Department of Chemical Engineering who's research interests are also in Bioengineering. The entire third floor of Othmer Hall is 14,000 ft^2 and is dedicated to Bioprocess and Bioengineering research.

The third floor is composed of two areas. The larger of the two areas is dedicated to the BPDF research development laboratories. This space is designed so that all of the process research and development scientist are located on one floor in a common integrated research environment. The objective was to create an environment that created the greatest level of interaction of personnel involved in all aspects of process research and development. The BPDF laboratories all share a common equipment corridor which maximizes the sharing of common equipment such as centrifuges, freezers, refrigerators, and shakers. Below are listed the different laboratories on the third floor.

Molecular Biology (MBL): A 990 ft^2 laboratory dedicated molecular biology and strain construction.

Fermentation Development Laboratory (FDL): A 1,200 ft^2 laboratory dedicated to bacterial and yeast fermentation research. The FDL will have 10 autoclavable BioFlo III and Bioflo 3000 bench-top 5 L fermentors (New Bruinswick Scientific) and 8 Bioengineering NLF 19 L steam-in-place fermentors. The FDL will have a 150 ft^2 microbiology support laboratory for growing inoculums and storing frozen seed cultures. All of the fermentors will be computer controlled and off-gas will be sent to an off-gas mass spectrometer for metabolic analysis.

Cell Culture Development Laboratory (CCDL): The CCDL is a new capability for the BPDF and has two different laboratories. One half of the space (750 ft^2) is dedicated to mammalian cell-line development and optimization and is outfitted with biosafety cabinets and CO_2 incubators. The purpose of this space is to develop mammalian cell clones suitable for transfer into a bioreactor and process scale-up. The other half of the facility (750 ft^2) is dedicated to bioreactors for scaling-up the process from 250 mL spinners to a 200 L bioreactor. The CCDL will have 8 by 250 mL DasGip computer controlled spinner system, 4 by 3.7 L computer controlled Bioengineering ALF bioreactors integrated to a Nova 400 bioanalyzer for automated sample analysis, one 19 L computer controlled Bioengineering bioreactor and one 200 L computer controlled Bioengineering bioreactor. The off-gas from the 3.7, 19 and 200 L bioreactors will be sent to an off-gas mass spectrometer. The primary function of the CCDL will be strain development, process research and development of production technology for humanized antibodies against bioterrorism agents in CHO cells.

Purification Development Laboratory (PDL): The PDL is a 1,500 ft^2 laboratory that has the responsibility of developing the recovery and purification process at the bench-scale (7.8 to 100 mL column size) and scaling-up the process to the 1 to 2 L column size and the production of small non-cGMP lots. The PDL will have "three research areas," bench-scale development area, central bench for routine assays, and the third is a small pilot plant area capable

of processing up to 0. 5 to 1 gram research lots of material.

***Media Preparation Area*:** The third floor will have a central media preparation area that will have two 26" x 36" x 38" Primus Autoclaves and a Miele Model G7827 large capacity glassware washer. There are two small lab areas will include a chemical hood, chemical storage cabinets and dry goods storage and laboratory space to prepare buffer and media.

Dark Room and Radioisotope Room. Two small 150 ft^2 labs are designated as a dark room and a radioisotope room.

cGMP Master Cell Banking Suite. A 860 ft^2 laboratory has been dedicated to cGMP production of Master Cell Banks (MCB) and Working Cell Banks (WCB). This suite will have approximately 360 ft^2 of Class 10,000 space with a 90 ft^2 Class 100,000 ante room for gowning. There will be a 600 ft Class 100,000 cGLP microbiology support laboratory which will have a 26" x 26" x 38" cGMP pass-thru autoclave and will provide all of the testing necessary to support MCB and WCB production. This GLP microbiology laboratory will also provide QC microbiology support for the cGMP pilot plants in the basement of Othmer Hall.

Three Bioengineering Research Laboratories. Three standard research laboratories are being constructed on the third floor. These lab spaces are planned for two faculty members in the Department of Chemical. One faculty member is working on antibody production and tissue engineering, while the second faculty member research interest is the production of complex proteins in transgenic animal. (Note: All animals for transgenic research will be housed in an animal facility in either the Departments of Animal Science or Veterinary and Biomedical Sciences on East Campus at the University of Nebraska-Lincoln.

Third Floor Office Area. The third floor will have office area for both staff and students. There will be an office area for the BPDF staff, which includes a conference room with both audio and visual conferencing capabilities.

Basement Area: A portion of the basement in Othmer Hall is being built out for storage space and office space for the cGMP pilot plant facility that is programmed for the basement and is described in the Appendix of the original proposal.

Additional Features of the Third Floor

Utilities. The third floor will have all of the standard utilities and additional utilities that are specific to the third floor. These include chilled water (10°C), clean steam distributed in 316L stainless steel piping, a biowaste kill system that will serve both the third floor and the basement, central gas storage room for distribution of oxygen, nitrogen, carbon dioxide and helium.

Electrical. The third floor will have 3 different types of power, uninterruptible power supply (UPS), emergency power (EP), and normal. The UPS system is located on the third floor electrical closet and is designated for critical systems, such as bioreactors, critical computer systems, and critical analytical equipment. The EP system is designed to come on 10 seconds after a power outage. Critical systems such as cold rooms, refrigerators, freezers, and equipment that can withstand a 10 second outage and still function will be on this system.

Security. The third floor will have a security system from AMAG Access Control Systems, the AMAG 625/675. This system will include electronic proximity detectors for all doors, video surveillance on all corridors, controlled access to the third floor, and an alarm system with motion sensors that are connected to the local police department which will indicate the location and time of unauthorized entrance or motion.

Third Floor Drawings. Drawings for the third floor and basement are provided in Appendix 1.

Production of S25 Antibody in CHO-DG44 Cells

Project Summary: The large scale production of humanized monoclonal antibodies (huMabs) with neutralizing activity against toxins (i.e. botulinum neurotoxin) has been identified as one of the highest priorities to counter bioterrorism (Blue Ribbon Panel on Bioterrorism: Sponsored by NIAID, February 4-5, 2002) as drugs for the treatment of botulism are currently unavailable. Recently, potent neutralizing monoclonal antibodies against botulinum neurotoxin serotype A (BoNT/A) were identified, characterized and further cloned to yield humanized Mabs against BoNT/A. It is our goal to develop and optimize a production strategy for the economical large scale production of these antibody based therapeutics for countering BoNT and other agents of biowarfare and bioterrorism. In pursuit of these objectives, we produced a humanized monoclonal antibody that has shown neutralizing activity against BoNT serotype A. We have developed several clonal cell lines with high levels of S25 monoclonal antibody production. pS25 was transfected into CHO-DG44 cells, which are negative for dihydrofolate reductase (dhfr(-)). This allows for gene amplification and increased antibody productivity through methotrexate addition. Much of the work to this point has focused on establishing high expressing methotrexate resistant cell lines. One of the original clonal cell lines produced 2.3 μg/ml of S25 antibody after three days of growth in adherent culture. This cell line was transferred to suspension culture by slowing weaning the cells from fetal bovine serum (FBS) and was able to produce 35 μg/ml of S25 antibody after 6 days in suspension culture. Milligram quantities of the S25 antibody have been produced and purified using a Protein A Sepharose Fast Flow resin. Numerous clones have been transferred to concentrations of methotrexate from 10 nM to 1μM. Clonal populations that resulted in high S25 antibody production underwent a second round of dilution cloning. To date, adherent cultures at 10 nM methotrexate resulted in S25 antibody titers as high as 22 μg/ml, and cultures at 100 nM methotrexate resulted in S25 antibody titers as high as 48 μg/ml. These cell lines are in the process of being transferred to suspension culture for further analysis. Several clonal cell lines (9) have been transferred to 1 μM methotrexate and will be cloned by limiting dilution and screened for S25 productivities. Once a high producing cell lines is established, the long-term stability will be analyzed and an optimized fed-batch production process will be determined. In addition, a new plasmid which allows for selection of S25 antibody producing clones has been constructed. This plasmid contains green fluorescence protein (GFP), and should allow for more efficient screening and development of antibody producing cell lines by flow cytometry.

Background: Botulinum neurotoxins (BoNTs) are listed as one of the 6 highest risk threats for bioterrorism by the Centers for Disease Control (CDC) due to their potency, lethality, and ease of production (1). Botulinum neurotoxin is expressed in the bacteria *Clostridium botulinum* and is one of the most poisonous substances known. There are seven BoNT serotypes (A-G), four of which (A,B,E,F) cause the human disease botulism(1,2). Botulism, which is often fatal, is characterized by flaccid paralysis requiring prolonged medical treatment. Currently, a toxoid against BoNT serotypes A to E can be used as a vaccine, but is dangerous to produce and represents only 5 of the 7 BoNT serotypes (3,4). Additionally, a recombinant vaccine using the purified protein product from genes encoding only the binding domain of BoNTs is under development. The recombinant proteins would not cause botulism since they lack the catalytic enzymes, but antibodies developed against these fragments could neutralize the corresponding BoNT serotypes (5). If a recombinant vaccine is successfully developed, it would likely be ineffective in post-exposure treatment of botulism due to the rapid onset of the disease. In addition, mass vaccination is unlikely since botulism is rare and any vaccination would prevent medical uses of BoNT. For this reason, it is necessary to develop post-exposure treatments against botulism.

Neutralizing antibodies can be used for both pre- and post-exposure treatment of botulism. Equine antitoxin and human immune globulin can be used to treat botulism, although antibody from these sources may pose a potential disease risk and would be difficult and expensive to produce on a large-scale (6,7). To solve these problems, monoclonal antibodies are being developed that could be used to treat botulism. Although no single antibody has been developed to neutralize botulism neurotoxin, a combination of monoclonal antibodies has been shown to neutralize BoNT serotype A (8). It was determined that a combination of three monoclonal antibodies could neutralize 450,000 50% lethal doses of BoNT serotype A. A plasmid encoding for one of the three monoclonal antibodies, S25, was provided by Dr. Jim Marks (University of San Francisco). The purpose of this project is to obtain an S25 cell line, and develop bioreactor conditions and purification strategies for the large-scale production of S25. This research will provide purified S25 antibody for research purposes and the methods to obtain this product may be applied to future monoclonal antibodies against BoNTs.

To obtain highly expressing cell lines, cells were transfected with a plasmid containing the light and heavy chains of the S25 monoclonal antibody, in addition to the gene dihydrofolate reductase (dhfr). Dhfr has been used in Chinese hamster ovary cells to increase production of recombinant proteins and monoclonal antibodies (9, 10, 11). CHO-DG44 cells, which lack the gene for dhfr, were obtained from Larry Chasin (Columbia University). This cell line allows for gene amplification through dhfr expression, which increases the number of copies of plasmid DNA. Methotrexate is used to bind the dhfr enzyme, which converts dihydrofolate to tetrahydrofolate. Dhfr deficient cells that incorporate plasmid containing dhfr can develop resistance to methotrexate addition by amplifying the dhfr gene. Typically, 100-3000 kb fragments of DNA are amplified in the gene amplification process, which is significantly longer than the dhfr gene (12). This typically results in co-amplification of the gene/genes of interest, the heavy and light chains of the S25 antibody in this case.

One problem associated with dhfr-mediated gene amplification is the stability of gene expression during the amplification process. Kim et al. 2001 found that only one out of 23 clones resulted in an increase in antibody productivity upon methotrexate addition. This clone showed approximately a 12.5 fold increase in antibody productivity (to 8 pg/cell/day) when subjected to 80 nM methotrexate in comparison to the parental cell line. Further addition of methotrexate resulted in a decrease in antibody productivity(12). The clonal cell lines were not re-cloned after methotrexate addition, and it is likely that clones obtained after transfecting with the S25 antibody plasmid will show significant variation after addition of methotrexate. To screen for cells with high antibody productivity, the S25 producing clones will be re-cloned in order to obtain cell lines with high antibody productivity. After the clones are isolated, the stability in the presence and absence of methotrexate will be examined.

It has been determined that a gradual increase in methotrexate addition is more effective in developing cell lines with high specific growth and production rates (13). As a result the methotrexate concentration was increased in a stepwise manner from 10 nM to 100nM to 1μM. In addition, the amplified gene location is critical in determining gene copy number and antibody production rate upon methotrexate addition. Telomeric type clones, where the amplified gene was near the telomeric region, were more stable and productive than other clones (14). This confirms that numerous clones need to be examined to isolate a cell line with high antibody productivity upon methotrexate addition. Typically, gene amplification occurs in a single chromosome at the location of the original integration site. In some cell lines, the amplified genes can be translocated to different chromosomes. Previous investigators have found that methotrexate addition resulted in 10-20 fold increase in expression levels from 1-3 to 30-50 pg/cell/day (15).

To improve the efficiency of gene amplification, a method of high throughput screening needs to be developed. This could be done in a 96 well plate format, but flow cytometry would allow for the screening of a significantly higher number of cells. Individual cells can be screened for antibody productivity using flow cytometry by trapping the antibody at the surface of the cell through formation of microbeads or use of a surface affinitiy matrix (16, 17, 18). It has been found that intracellular protein content typically correlates with cell productivity (19, 20). In addition, various investigators have optimized transfection and screening procedures using the reporter proteins, green fluorescence protein (GFP) and β-galactosidase (21, 22). It is possible to co-express GFP, along with the protein of interest, and then use GFP to screen for high producing clones (23). An additional application is to target the gene of interest to a particular location in the DNA. The cells are transfected with a plasmid containing GFP, along with a recombinase target site, and are then screened the for GFP production. This is followed by targeting the protein of interest to the site where the GFP was integrated (24). In the hopes of obtaining CHO cell lines with increased S25 antibody productivity, we have constructed a plasmid that allows for the co-expression of GFP and the S25 antibody.

Results, Discussion and Current Progress

Cell Line, Media, Transfection and Expression Vectors:The CHO-DG44 cell line, obtained from Dr. Larry Chasin of Columbia University, was used as a host because it is dhfr negative and can be amplified for gene expression upon methotrexate addition. The host cell line was maintained in α-MEM media (Invitrogen) supplemented with 8% fetal bovine serum (FBS)(Invitrogen). The cells were transfected with plasmid pS25 which was generously provided by Jim Marks of the University California San Francisco. The pS25 plasmid was constructed by inserting the light and heavy chain IgG genes against BoNT serotype A, along with the gene for dhfr into the plasmid pcDNA3.1(+) (Invitrogen)(Figure 1).

The CHO-DG44 cell line was transfected with the pS25 plasmid using Lipofectamine 2000 (Invitrogen). Briefly, cells were seeded with 0.5 ml in 24 cell plates at 2 x 10^5 cell/ml in α-MEM media with 8% FBS and grown overnight. 1 μg plasmid DNA and 0.5-5.0 μl Lipofectamine 2000 were combined in 0.1 ml Opti-MEM media (Invitrogen) and allowed to equilibrate for 20 min. 0.5 to 2 ml of Lipofectamine 2000 proved to be optimal. Plasmid DNA was added to the transfection mix either uncut or linearized with NruI. This site was chosen since it would place the amplifiable gene (dhfr) between the heavy and the light chains. The DNA/Lipofectamine 2000 solution was added to the 24 well plates and the plates were incubated at 37°C overnight. Stably transfected cells were selected in α-MEM media lacking ribonucleotides and deoxyribonucleotides (α-MEM(-)) and with 8% FBS, which prevents cells lacking dhfr from growing. Cells were passaged several times and individual clones were obtained by dilution cloning at 0.5 cells/well in 96 well plates. Samples were taken from wells containing growing cells after 16-18 days. The antibody concentrations were determined using an ELISA specific for the Fc portion of a human heavy chain of IgG. Sixteen of the cell lines showed antibody titers greater than 0.1 μg/ml, while one

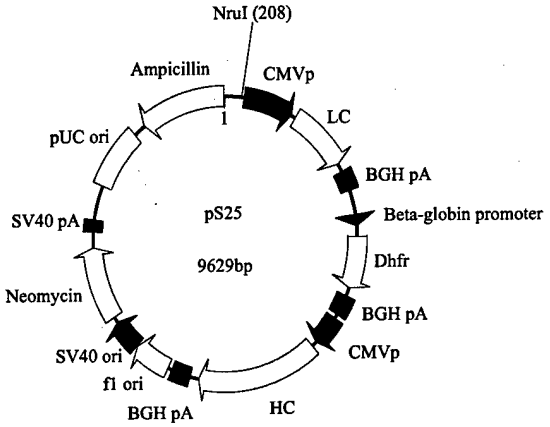

Figure 1. Diagram of plasmid pS25 (Obtained from Dr. Jim Marks). Plasmid contains the light (LC) and heavy chain (HC) of S25 antibody against BoNtT/A. It also contains dhfr as a selectable marker, which can be used for gene amplification.

showed a concentration of greater than 2 μg/ml (Figure 2). This clone (CHO-DG44 S25 #56) ended up having the highest expression level throughout the selection process. Presence of the light chain was later confirmed by both Western blotting and a human kappa chain specific ELISA. Cells with an antibody concentration of greater than 0.1 μg/ml were transferred first to 24 well plates and then to 25 cm^2 T-flasks. Cells were seeded at 2 x 10^5 cells/ml in 9 ml α-MEM(-) supplemented with 8% FBS in a 25 cm^2 T-flask. Nine clones showed expression levels greater than 0.5 μg/ml after three days in adherent cultures in the 25 cm^2 T-flasks (Figure 3). These nine clones were transferred to methotrexate for gene amplification and into CHO-S-SFM II in order to ensure culture stability and productivity is suspension culture. The cell line CHO DG44 S25 #56 had light and heavy chain concentrations greater than 2 μg/ml and was therefore used for the production of S25 antibody in suspension culture.

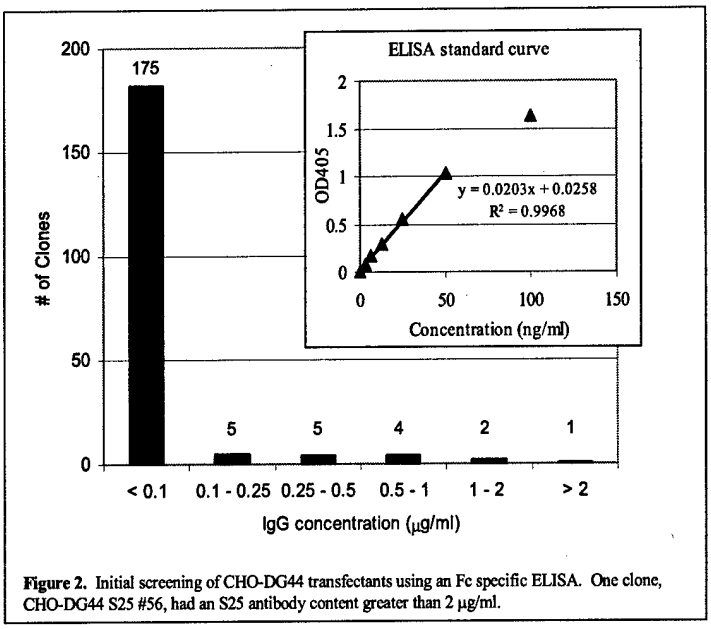

Figure 2. Initial screening of CHO-DG44 transfectants using an Fc specific ELISA. One clone, CHO-DG44 S25 #56, had an S25 antibody content greater than 2 μg/ml.

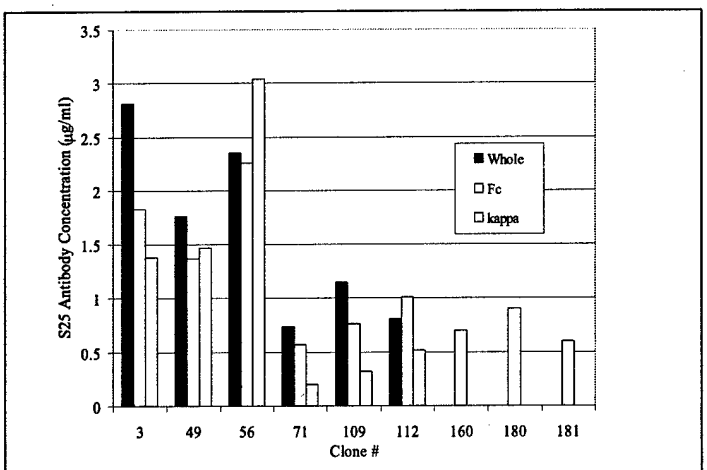

Figure 3. Comparison of S25 antibody production in various CHO-DG44 S25 clones. Clones 3, 49 and 56 had the highest levels of expression. Clone #56 was chosen for initial production of S25 antibody. Whole IgG and kappa concentrations were not determined for clones 160, 180, and 181. The concentration is the total amount of S25 antibody based on either a whole molecule, Fc specific or kappa specific ELISA.

ELISA: The concentration of the whole antibody, as well as the concentration of the light and heavy chain portions were determines by an enzyme-linked immunosorbent assay. Affinity purified rabbit anti-human IgG antibodies were diluted to 5 μg/ml in coating buffer consisting of 100 mM $NaHCO_3$ and 100 mM NaCl (pH = 9.3). One hundred μl diluted antibody was added to 96 well plates (Nunc) and incubated overnight at 4°C. The plates were washed twice in Tris buffer (20 mM Tris-HCl, 50 mM NaCl, pH = 7.2) containing 0.1% Tween 20 and then twice in Tris buffer alone. Blocking buffer (Tris buffer with 0.5% BSA) was added to the 96 well plates and incubated at 37°C for 1 h. Supernatant samples were diluted in the blocking buffer and samples were loaded into the 96 well plates in triplicate. Plates were incubated for 1 h at 37°C and the washing procedure was repeated. One hundrec μl of a goat anti-human IgG-HRP conjugate antibody diluted to 0.5 to 2 mg/ml in the dilution buffer was added to the plates. The plates were incubated for 1 h at 37°C and the washing procedure was repeated. Lastly, 100

ml of 50 μg/ml ABTS in ABTS buffer (Roche) was added to the plates. The absorbance was determined at 405 nm using a ELx800 plate reader (Bio-Tek). This procedure was used for whole antibody, heavy chain (Fc specific), and light chain (κ specific). Whole, Fc and kappa rabbit anti-human IgG coating antibodies and whole, Fc and kappa goat anti-human IgG-HRP conjugated antibodies were used in the ELISAs (Sigma).

Transfer to Suspension Culture: After screening the clones for antibody production, all nine clones that reached 0.5 μg/ml antibody after three days were transferred to suspension culture. These clonal cell lines were frozen and will be analyzed for antibody production along with the methotrexate gene amplified cell lines, once the methotrexate resistant clones are transferred to suspension culture. To transfer cells to suspension culture, the cells were grown in 50 ml spinner flasks (Wheaton) with decreasing concentration of FBS. Initially the cells were seeded in the spinner flasks at 2-3 x 10^5 viable cells/ml in CHO-S-SFMII media (Invitrogen) containing 1% FBS, and were passaged every 2-4 days into fresh media containing decreasing amount of FBS. After 8-10 passages the cells were frozen in α-MEM media with 10% FBS and 10% dimethylsulfoxide (DMSO)(Fisher) at a cell density of 10^7 cell/ml. The cells were slowly frozen at -80°C in 1.5 ml aliquots and were stored in liquid nitrogen for later use.

Growth of Cells in Suspension Culture: The CHO-DG44 S25 #56 cell line was grown in batch culture in order to demonstrate antibody production in suspension culture and to produce a sufficient amount of S25 antibody for research purposes. Frozen cells were taken from liquid nitrogen and were resuspended in 40 ml CHO-S-SFMII, resulting in a seeding density of 3-4 x 10^5 cells/ml. The spinner flasks were incubated at 37°C and 5% CO2. The cells were fed every 3-4 days for several passages and were then seeded at 2 x 10^5 viable cell/ml in 350 ml CHO-S-SFMII media in a 1L controlled spinner flask. The dissolved oxygen (DO), pH and temperature were controlled using a DAS-GIP cellferm-pro control system. The pH was controlled by addition of CO_2 and 1M NaOH. Samples were taken every day and viability and cell density were determined by trypan blue exclusion and counting on a hemocytometer. Cell suspensions were centrifuged at 1200 rpm for 5 min and supernatant samples were frozen for later analysis. To obtain purified S25 antibody, the spinner flasks were harvested after the viability began to decline on day 6. The CHO-DG44 S25 #56 cell line reached a cell density of 4 x 10^6 cells/ml in CHO-S-SFM II media (Invitrogen) after 5 days in suspension culture (Figure 4). The viability remained above 90% until day 6 at which point it dropped to 80% (Figure 5). At this point the spinner flasks were harvested by centrifugation and the S25 antibody was purified. Since the DAS-GIP controlled spinners are able to monitor oxygen consumption, a drop in the oxygen uptake rate at approximately 130 h was the first sign that the cells were beginning to die (Figure 6). After growing the CHO-DG44 S25 #56 cell line for 6 days in batch culture, the S25 antibody concentration reached a average of 35 μg/ml in CHO-S-SFM II media (Table 1). The mean specific antibody productivity was 3.1 pg/cell/day, which is similar to that found for other recombinant antibodies (15).

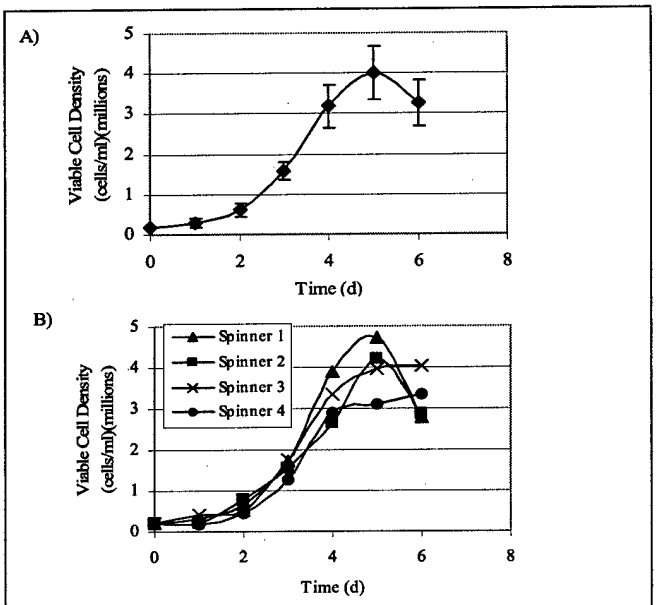

Figure 4. Growth of CHO-DG44 S25 #56 cells in suspension culture in DAS-GIP bioreactors. A) Mean, B) Individual spinner flasks.

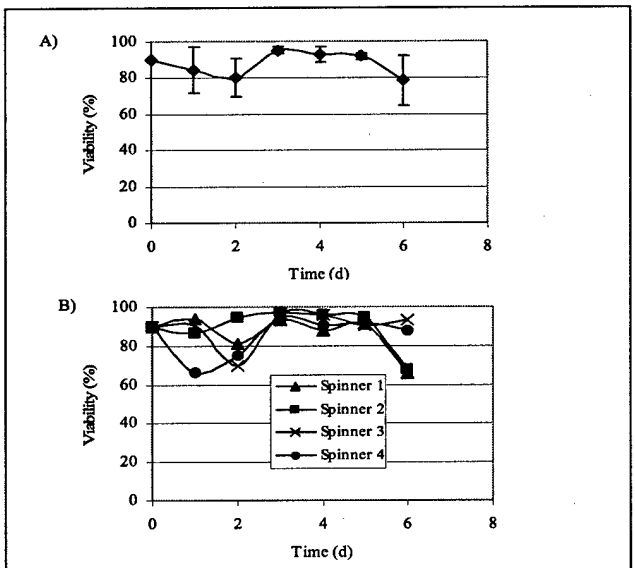

Figure 5. Viability of CHO-DG44 S25 #56 cells in suspension culture. A) Mean, B) Individual spinner flasks.

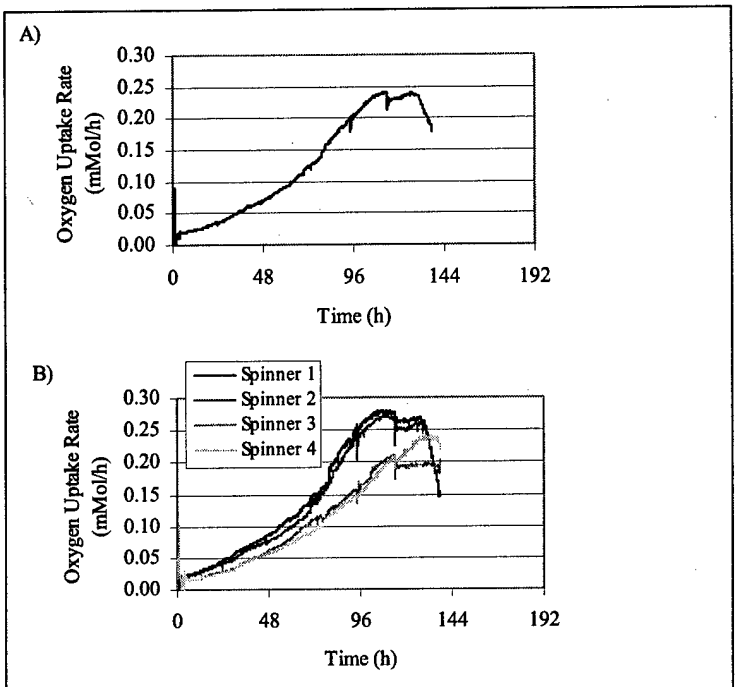

Figure 6. Oxygen uptake rate of HCO-DG44 S25 #56 cells in suspension culture. The drop in OUR was the first sign that the viability in the spinner was beginning to decline.

Spinner Flask #	Final S25 antibody concentration (μg/ml)	Specific S25 Productivity (pg/cell/d)
1	52.5	4.1
2	32.8	3.0
3	20.7	1.7
4	34.0	3.5
Mean	**35.0**	**3.1**

Table 1. S25 antibody final concentrations after growing for 6 days in suspension culture. The average specific productivity was similar to that observed for other recombinant antibodies (15).

Protein Purification: The supernatant from the CHO-DG44 S25 #56 cells was harvested after 6 days in batch culture. The S25 antibody in the spinner flasks was purified according to Figure 7. The supernatant from the spinner was harvested by centrifugation at 300xg for 5 min. The supernatant was then diafiltered with 20 mM Na_2HPO_4 / 150 mM NaCl (pH=7.3) using a Pellicon XL50 ultrafiltration device containing a 0.005 m^2, 10 kD Biomax membrane. The diafiltered sample was loaded onto a column by gravity containing Protein A Sepharose Fast Flow resin (Pharmacia). Two ml of resin was used, which provided a bed height of about 2 cm. After loading the sample onto the column, the column was washed 2 times with 25 ml Na_2HPO_4/ NaCl. The purified humanized antibody was eluted in 20 mM sodium citrate (pH=3.0). The pH of the elution was immediately increased to 7 using 500 mM Tris buffer (pH=9.0). The antibody was then concentrated and transferred into PBS by 10 fold diafiltration using a Pellicon XL50 ultrafiltration device. The S25 antibody was quickly frozen in liquid nitrogen at 1 mg/ml (BCA Assay) and was stored at -80°C for long-term storage.

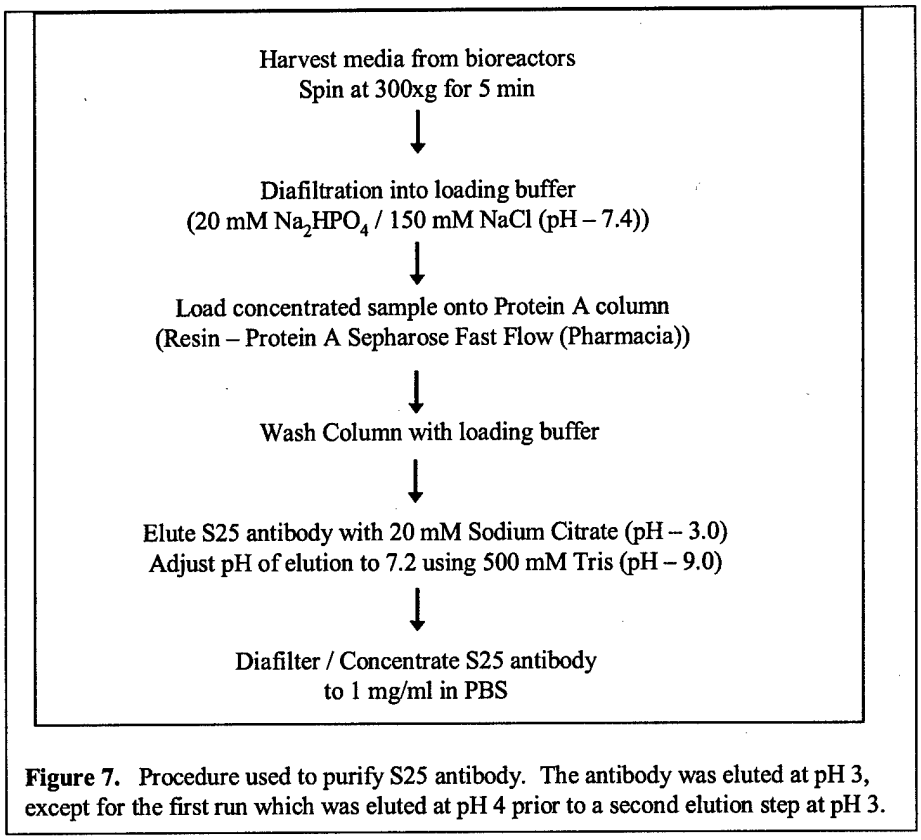

Figure 7. Procedure used to purify S25 antibody. The antibody was eluted at pH 3, except for the first run which was eluted at pH 4 prior to a second elution step at pH 3.

After purification, protein samples were run on 12% polyacrylamide gels with a tris/glycine running buffer. In an initial run the S25 antibody was eluted at pH 4, followed by a second elution at pH 3. In the coomassie stained gel (Figure 8A), faint bands for the heavy and light chains can be seen in lanes 6 and 7 (culture supernatant

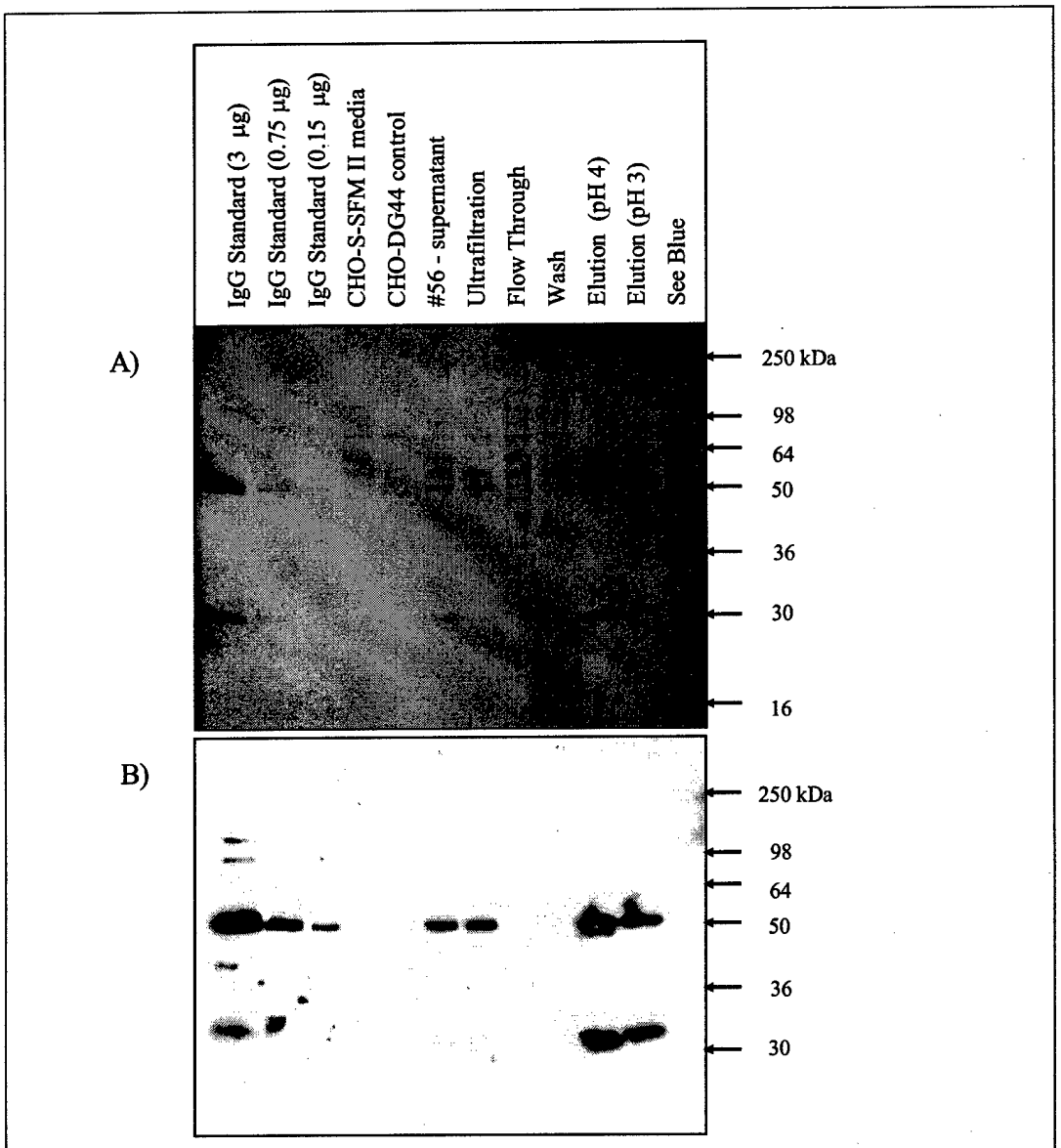

Figure 8. Anlysis of samples from S25 antibody purification using Protein A sepharose fast flow resin. A) Coomassie stain, B) Western blot. Bands corresponding to the heavy and light chains of S25 IgG are clearly evident in the culture supernatant, ultrafiltration sample and elution fractions. Lanes 10 and 11 show that the elution from the column is very pure. Both of these elutions were pooled for the final product

from the CHO-DG44 S25 #56 cell line and the ultrafiltration sample). Three µg of total protein were loaded into each lane of the coomassie stained gel, except for the CHO-S-SFM II media, the CHO-DG44 control and the elution sample at pH 3. The concentrations of these samples were less than 150 µg/ml, and therefore, 20 µl of each was loaded. The gel shows that little antibody was lost in the flowthough and wash steps and that the elutions at pH4 and pH 3 were very pure. These results were confirmed by Western blotting using a goat anti-human IgG (whole molecule specific)(Sigma)(Figure 8B). The Western and Coomassie figures were from duplicate gels. The purification procedure was repeated 3 more times, using supernatant from different reactor runs each time. Samples were once again analyzed by coomassie staining and Western blotting. For these runs a single elution step at pH 3

was used. All of the eluted samples were pure and band size was consistent from run to run (Figures 9 and 10). The Coomassie stained gel and the Western are not exact duplicates. Non-reducing gels were also run in order to ensure that the heavy and light chains formed complete antibody (Figure 11). Although the coomassie is a little light and the Western was overloaded, it can be seen that lane numbers 4 and 5 show full S25 antibody that correspond to the purified S25 antibody. There was no unbound light or heavy chain detected in the purified S25 antibody or supernatant samples. Lanes 12 and 13 show reduced samples of purified S25 antibody and culture supernatant for comparison. Included on the gel, in lanes 8-11, are samples taken from Picchia cultures that express the S25 antibody. The concentration of the samples was too low to detect on the coomassie stains. Antibody expression is evident for one clone on the Western blot, although there are numerous bands in comparison to the product produced in CHO. Formation of complete antibody needs to be confirmed.

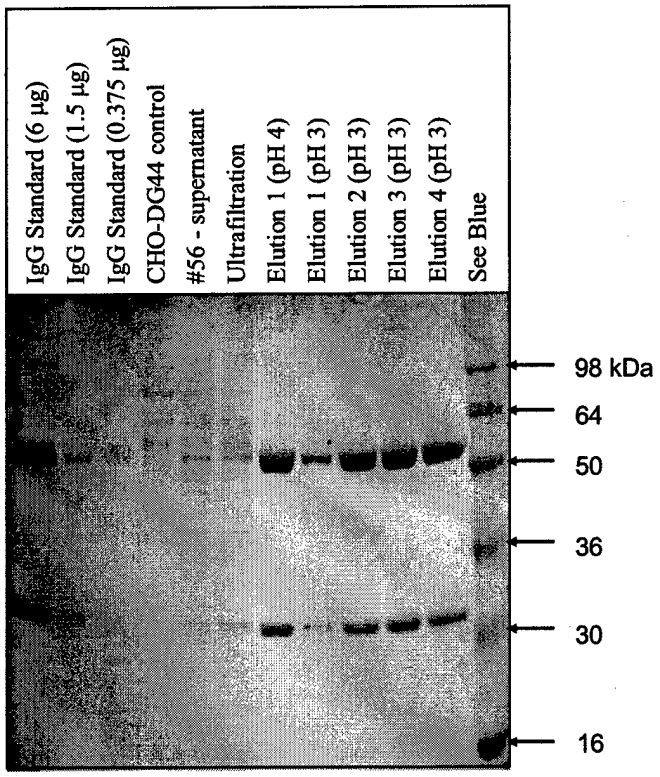

Figure 9. The purification of S25 antibody was repeated on 4 different spinner flasks. The purified samples were consistent from run to run.

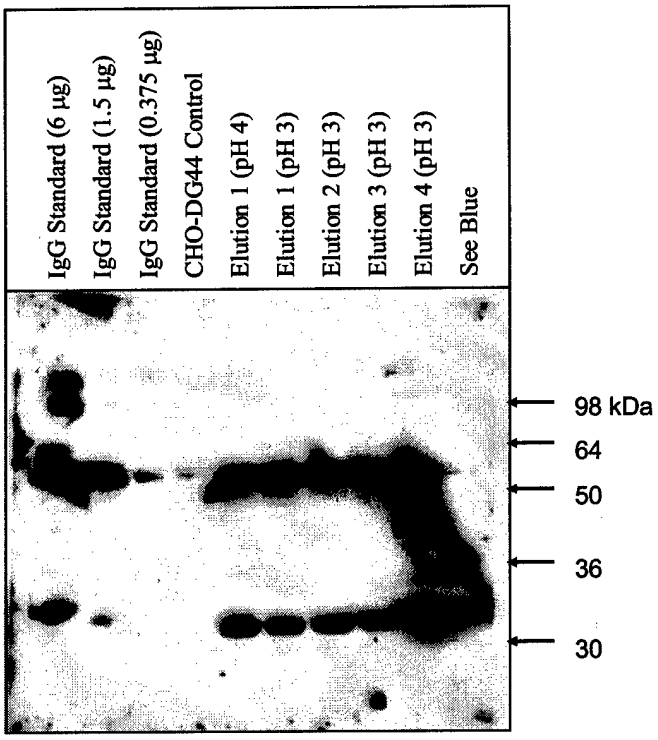

Figure 10. Western blot of elution fractions from S25 antibody purification

The purification of S25 antibody with Protein A Sepharose Fast Flow provided a 76% yield (Table 2). In the initial purification, there was 15.2 mg of antibody in the supernatant at a concentration of 52.5 µg/ml. There was little loss of antibody as the result of the ultrafiltration into loading buffer (20 mM Na_2HPO_4 / 150 mM NaCl). The S25 antibody was purified 11 fold and appears to be greater than 95% pure as determined by coomassie staining. The total protein concentrations were initially determined by Bradford assay using bovine serum albumin (BSA) as a standard (Table 1). There was some discrepancy between the results of the Bradford assay and the BCA assay which was later used to quantify concentration of the final purified product. The concentration of the elution samples was approximately 80% higher when determined using the BCA assay with human IgG as a standard. This caused the total protein concentration, determined using the Bradford assay, to be lower than the IgG concentration determined by ELISA. These samples were sent to Dr. Len Smith for further analysis.

Bradford / BCA Assay: The total protein content for the purified humanized antibody and the culture supernatant were determined using either a Bradford assay (Coomassie reagent, Sigma) or BCA reagent (Pierce). For the Bradford assay, a 1 ml sample was mixed with 1 ml Bradford Reagent (Sigma). BSA was used as a protein standard. The samples were incubated for 30 min at 37°C and the absorbance at 595 nm was determined on a spectrophotometer. The BCA assay was used to determine the final concentration of the S25 antibody product. For the BCA assay, 50 µl sample or standard was mixed with 1 ml BCA reagent. Human IgG was used as the standard. The samples were incubated for 30 min at 37°C and the absorbance at 562 nm was determined on a spectrophotometer.

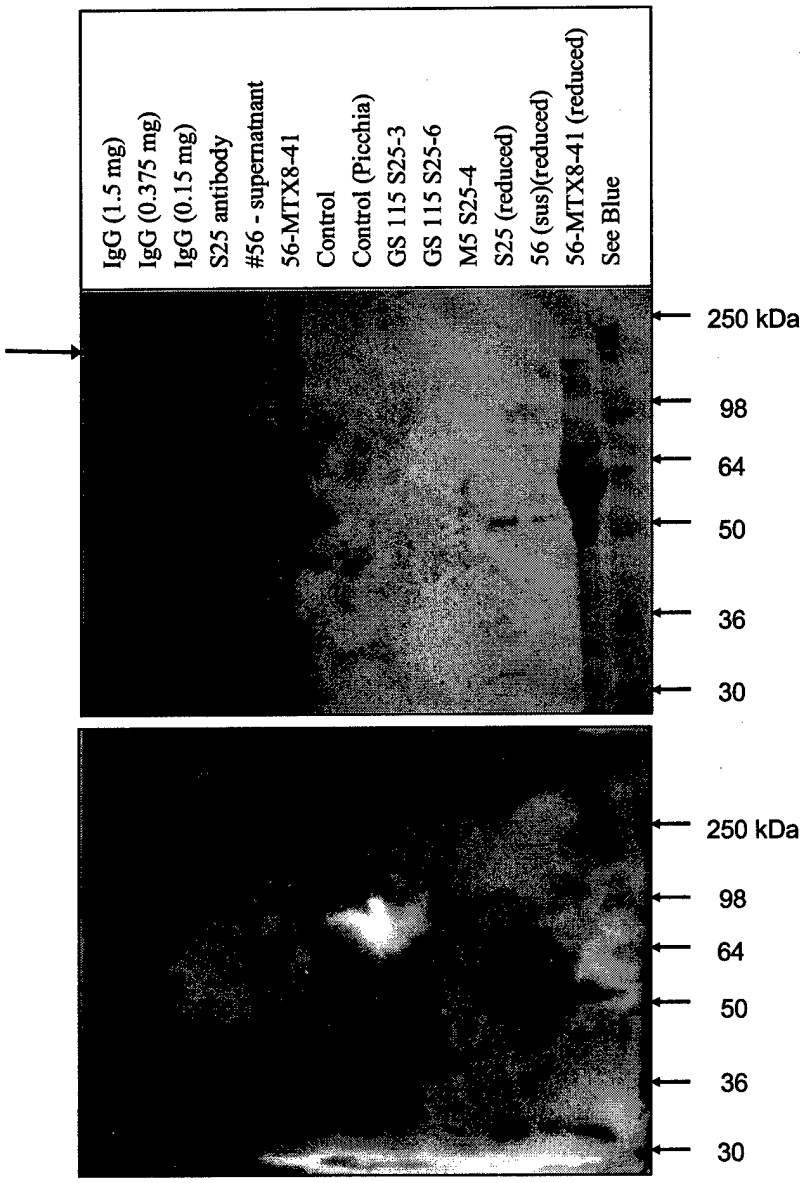

Figure 11. Coomassie stain and Western blot of purified S25 antibody. The first 11 lanes were non-reduced resulting full intact antibody running at ~150 kDa (See the arrow on the left). Reduced samples are shown in lanes 12 and 13.

Table 2. Detailed summary of the purification of S25 antibody. 76% of the antibody was recovered in the two elution steps.

	Volume (ml)	S25 IgG (μg/ml)	Total S25 IgG (mg)	Total Protein Conc. (μg/ml)	Total Protein (mg)	Yield (%)	Pruification Factor (Fold)
Supernatant	290	52.5	15.2	374	112.2	100	1
Diafiltration	39	373	14.6	2689	104.9	96	1.03
Flow Through	39	3.6	0.14	1444	56.3	0	-
Wash 1	25	2.6	0.07	207	5.2	0	-
Wash 2	25	-	-	3.5	0.1	-	-
Elution 1 (pH 4)	27.1	347	9.4	238	6.4	61.8	10.8
Elution 2 (pH 3)	22.6	96.5	2.2	62	1.4	14.5	11.6
Elute (total)	49.7	233	11.6	157	7.8	75.6	11.0

Western Blotting: Supernatant samples from growing cultures were obtained by centrifugation at 1200 rpm for 5 min. Samples were diluted in phosphate buffered saline (PBS) to 60 μl and 20 μl loading buffer (0.5 M Tris-HCl, 20% SDS, 40% glycerol, 10% β-mercaptoethanol, 0.1% bromophenol blue) was added. Samples were boiled for 2 min and resolved on a 10-12% Tris-glycine polyacrylamide gel (Invitrogen). The gels were run for 2-4 h at 125 V in an XCell SureLock Mini-Cell (Invitrogen) containing Tris/glycine/SDS buffer. The gels were transferred to nitrocellulose in an XCell SureLock Mini-Cell module for 6 h at 25V in tris-glycine transfer buffer containing 20% methanol. Blots were blocked with 5% nonfat dry milk in TD buffer for 2 h at room temperature. Recombinant humanized monoclonal antibody was detected by incubating with 0.5 mg/ml goat anti-human IgG (whole molecule specific)(Sigma) in 5% nonfat dry milk in TD buffer for 1 h at room temperature. The protein bands were detected by incubating with ECL (Amersham) and exposing to film. Human IgG (Sigma) was used as a positive control. For non-reducing gels, the loading buffer lacked SDS and β-mercaptoethanol.

Gene Amplification: After stable clonal cell lines were established, the level of S25 antibody production was increased by gene amplification. Methotrexate (Sigma) was added to the selection media in stepwise increments from 10 nM to 1 μM. The nine clonal cell lines that showed S25 antibody concentrations greater than 0.5 μg/ml, including CHO-DG44 S25 #56, were transferred to 10 nM methotrexate in α-MEM(-) with 8% FBS. After several passages, these clones were transferred to 100 nM methotrexate and then to 1μM methotrexate. At each step, the cell line CHO-DG44 S25 #56 was subjected to dilution cloning in order to obtain clones with elevated levels of S25 antibody production. All nine clones have been frozen at methotrexate concentrations of 10 nM and 100 nM for

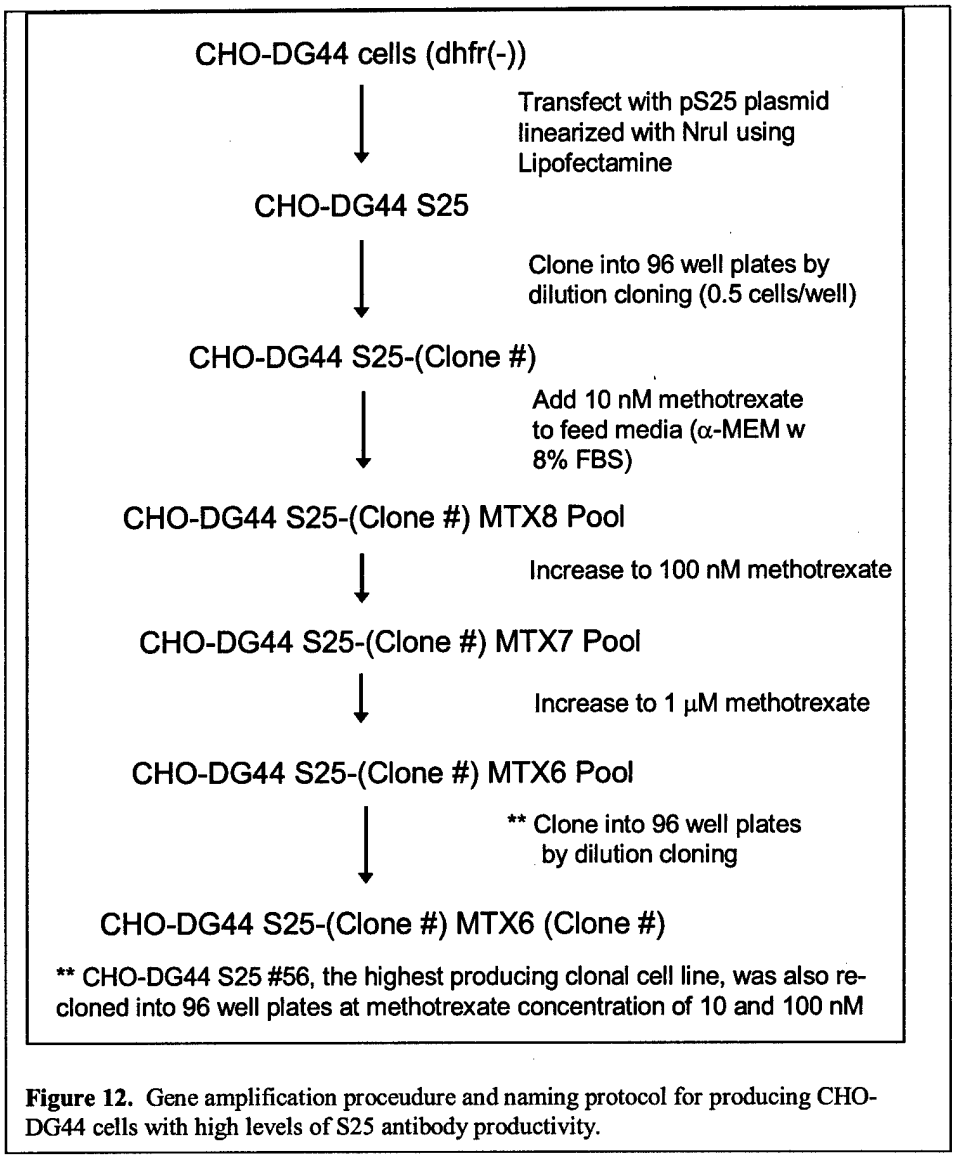

Figure 12. Gene amplification proceudure and naming protocol for producing CHO-DG44 cells with high levels of S25 antibody productivity.

later use. Several of the clones are growing in 1 μM methotrexate, while others are still in the process of being transferred. A details diagram showing the gene amplification procedure and a naming procedure for the different cell lines is provided (Figure 12). The highest expressing pools, of the other eight cell lines, will be re-cloned after transferring the cells to 1 μM methotrexate.

The cell line CHO-DG44 S25 #56 was re-cloned at methotrexate concentrations of 10 nM, 100 nM, and 1 μM. CHO-DG44 S25 #56 cells grown in adherent culture reached an S25 antibody concentration of 2.3 μg/ml after 3 days of growth. After transferring the cell line to 10 nM, 100 nM, and 1 mM methotrexate the resulting pools of cells reached S25 antibody concentrations of 2.8, 5.3 and 2.7 μg/ml, respectively (Figure 13). There was little increase in the antibody production of the pool of cells upon methotrexate induced gene amplification. These cells were then re-cloned to isolate a single cell with an increase in antibody production. Forty to Sixty clones at each methotrexate concentration have been examined. The highest producing clones gave S25 antibody concentrations of 22 and 48 μg/ml at methotrexate levels of 10 and 100 nM, respectively (Figure 14). The individual clones showed an increase in antibody titer of

greater than 10 fold upon methotrexate gene amplification. At the highest methotrexate concentration tested (1 μM), none of the clonal cell lines produced elevated levels of S25 antibody. At each step of the gene amplification process, fewer and fewer cells showed elevated S25 antibody production. Non-producing cells began to take over the culture. In order to solve this problem, we have begun transferring the CHO-DG44 S25 MTX7

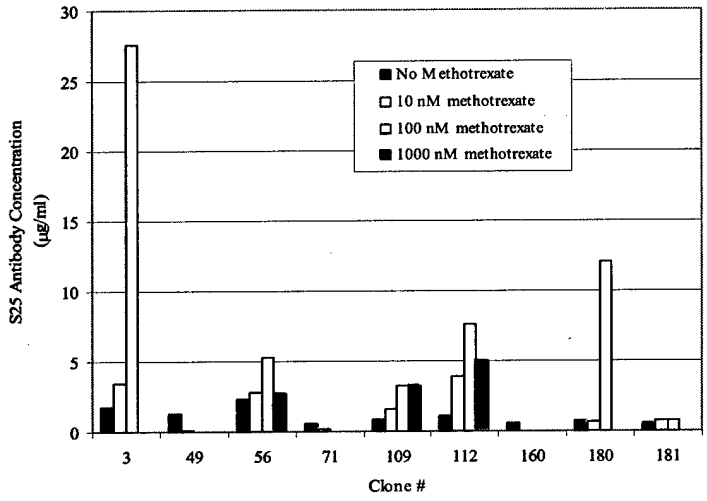

Figure 13. Effect of dhfr mediated gene amplification on various CHO-DG44 S25 clones. Clones 3, 56, 109,112, and 180 showed increased S25 antibody production upon methotrexate addition up to 100 nM methotrexate. The pools began to lose expression as methotrexate was further increased. It appears that some cells in the population lose expression, while other increase expression with an increase in methotrexate. It may be necesssary to concentrate cells with high expression levels as the level of methotrexate is increased.

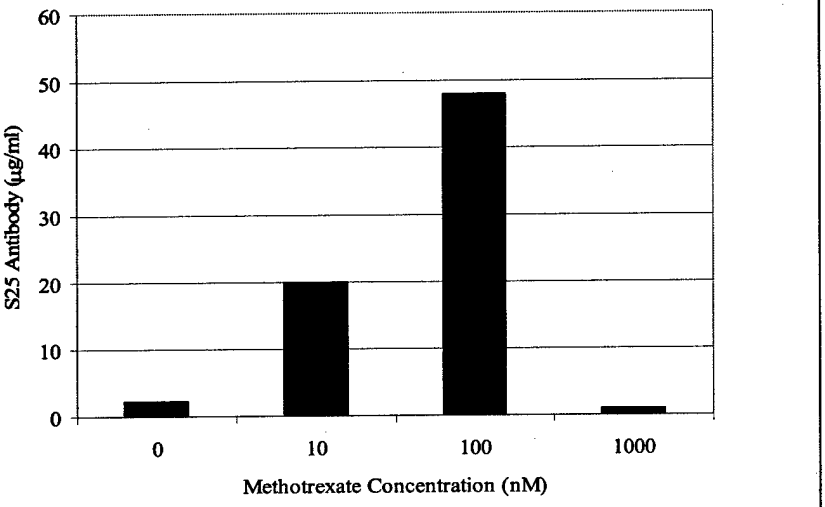

Figure 14. S25 antibody concnetration of CHO-DG44 S25 #56 clones at different levels of methotrexate. None of the 40 clones tested at 1000 nM methotrexate produced antibody concentrations greater than 2 µg/ml, the exact level was not determined.

clones to increasing levels of methotrexate from 200 to 1000 nM. It should be noted that in addition to CHO-DG44 S25 #56, the cell line CHO-DG44 S25 #112 was re-cloned at a methotrexate concentration of 1 µM and resulted in a S25 antibody concentration of 15 µg/ml after 3 days in culture.

Nine CHO-DG44 S25 clones have been transferred to increasing concentrations of methotrexate for gene amplification. Five of the nine clones showed a significant increase in S25 antibody production with methotrexate addition, the rest did not (Figure 13). These five clones are being examined to find the highest producing cell lines. Prior to the re-cloning step, several clonal pools showed a significant increase in S25 antibody productivity. CHO-DG44 S25 #3, CHO-DG44 S25 #112 and CHO-DG44 S25 #180 cells grown in 100 nM methotrexate produced antibody concentrations of 27.6, 12.0, and 7,6 µg/ml after three days in adherent culture, respectively. These cell lines have been stored frozen and may be re-cloned to isolate specific cell lines with increased S25 antibody productivity. Figure 10 summarizes the current progress in obtaining cell lines with high S25 antibody productivity.

It should be noted that the plasmid pcDNA-S25 was not linearized with NruI prior to transfecting clones 1-48. Clone #3 was the only clone produced with non-linearized plasmid that had an S25 antibody concentration greater than 0.5 µg/ml. At a concentration of 100 nM methotrexate, the CHO-DG44 S25 #3 MTX7 pool had the highest S25 antibody concentration. This suggests that while NruI linearized plasmids result in a higher number of producing cell lines, integration at another site may allow for higher stability during gene amplification. Possibly the plasmid enters the chromosome in a more stable orientation. The problem is that if a gene in the plasmid is disrupted when integrated into the DNA of the cell, that gene would not be expressed. Since only one cell line was generated, it is difficult to determine the reason why this cell line is more stable during gene amplification.

Construction of GFP (Green Fluorescence Protein) Plasmid: To decrease the time and effort required to isolate high expressing gene amplified cell lines, we have constructed the plasmid pS25-GFP, containing GFP-zeocin in place of the neomycin gene in the pS25 plasmid (Figure 15). This plasmid will allow for screening of amplified cells by flow cytometry, providing us the ability to analyze a significantly higher number of cell lines. This also gives us the ability to concentrate cells with high GFP production during the gene amplification process. Cells with high GFP content will likely have high antibody productivities. The insertion of GFP-zeocin still needs to be confirmed by DNA sequencing, and production of GFP and S25 antibody needs to be established by transfecting CHO-DG44 cells and generating stable clones.

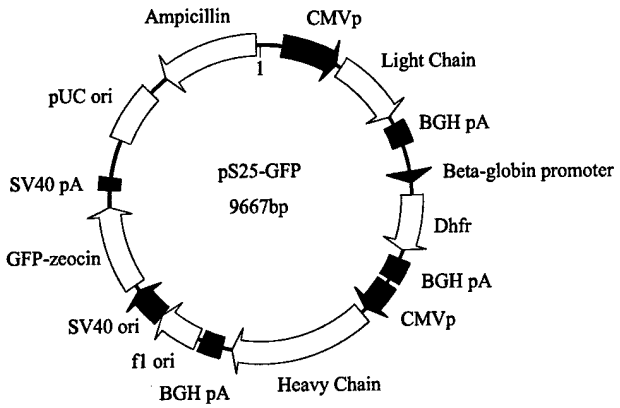

Figure 15. The plasmid pS25-GFP has been constructed for screening S25 expressing clones by flow cytometry. After some manipulation, the neomycin gene in pS25 was replaced with GFP-zeocin. The sequence and expression of this plasmid needs to be confirmed.

Media Development: In addition to generating cell lines that produce S25 antibody, work is being done to develop media and reactor conditions for large-scale production of antibody to be used as therapeutics. CHO-DG44 cells were grown in suspension culture to investigate the effect of pH on cell growth. Cells were seeded in controlled DAS-GIP spinner flasks at 2 x 10^5 viable cells/ml in CHO-S-SFM II media containing HT supplement (hypoxanthine / thymidine). The cells reached a peak in cell density after 4-5 days in culture with the highest being 1.8-1.9 x 10^6 cells/ml at a pH of 7.0 or 7.2. The viable cell density dropped off quickly at elevated pH (Figure 16A). At pH 7.4 the viable cell density dropped off

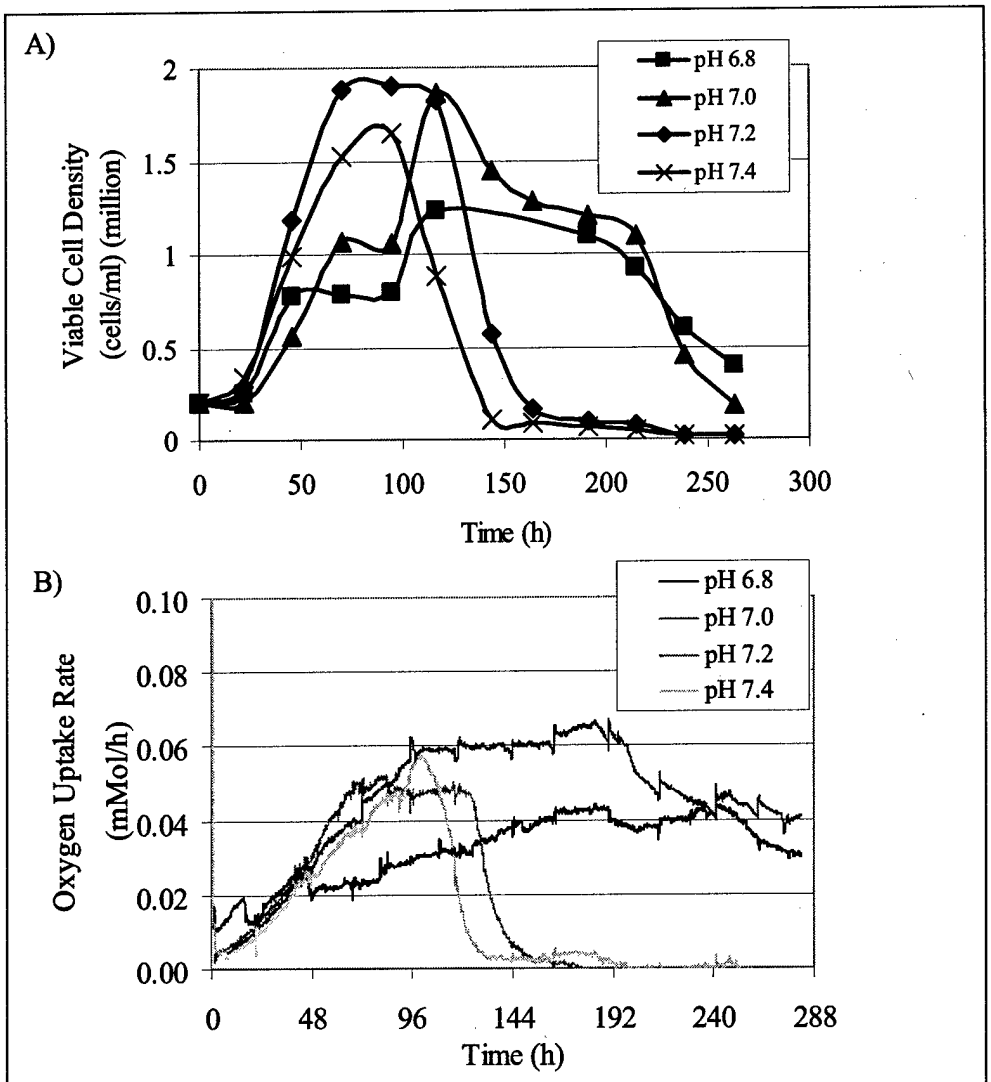

Figure 16. Grow of CHO-DG44 cells at various levels of pH. Cells grown at pH 7.2 and 7.4 had a quick decline in viable cell density after 100-140 h. Cells at pH 7.0 grew quickly and had a slower decline in viable cell density. A) Viable Cell Density, B) Oxygen Uptake Rate.

after approximately 100 h and at pH 7.2 the viable cell density dropped off after 130 h. From this data, along with the oxygen uptake data (Figure 16B) it is evident that the optimal pH is 7.0. The results of this experiment were somewhat expected since this experiment was done to become familiar with the DASGIP control system. However, it was surprising how quickly the viable cell density dropped off at elevated pH and is being mentioned so that this is avoided in the future.

Additional work has been done to grow cells in a chemically defined low protein media. This is important for the large scale production of therapeutic proteins. It will be necessary to optimize the cell culture media as additional antibodies and other therapeutic proteins are developed. The cells were seeded at 2 x 10^5 cells/ml and were grown in a

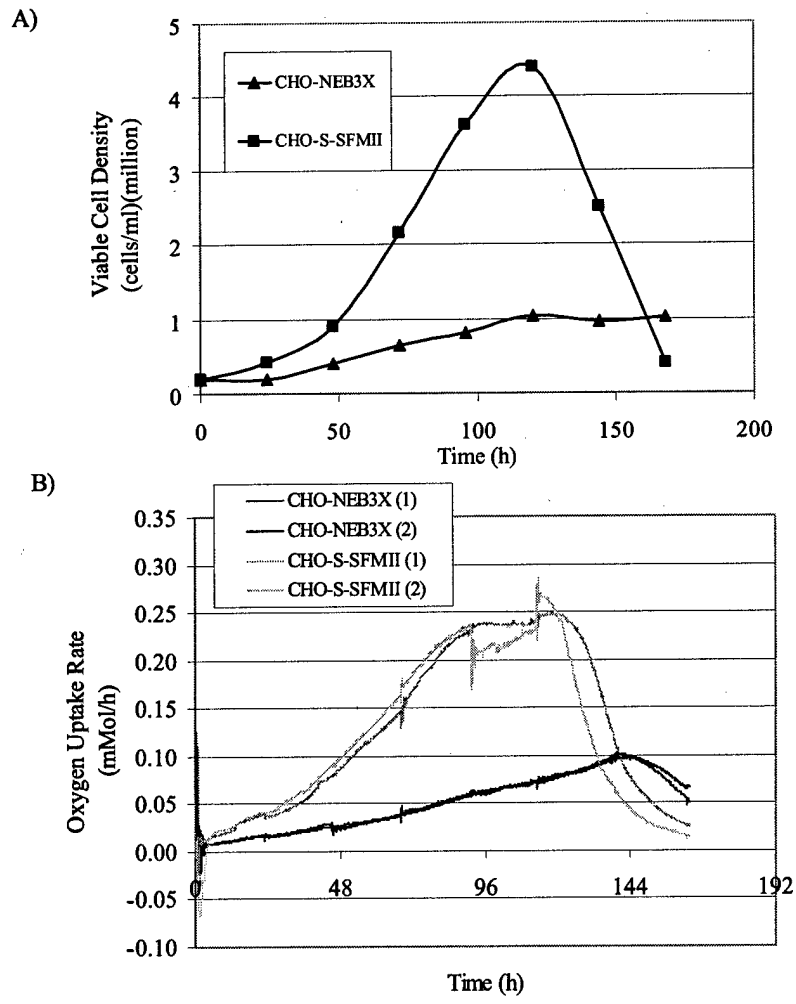

Figure 17. Grow of CHO-K1 cells in CHO-S-SFM II, a commercially available low protein media, and CHO-NEB3X, an initial chemically defined media being developed in our lab. There is a need to move towards chemically defined media in the production of proteins in mammalian cell culture. Towards this pursuit, we a developing media for large scale production in our laboratory

CHO-S-SFMII, as well as a supplemented basal media. The CHO-DG44 cells grown in CHO-S-SFMII reached a viable cell density of 4.3×10^6 viable cells/ml compared to 1.0×10^6 in CHO-NEB3X media. Since CHO-S-SFM II is not a defined media it is not suitable for production of proteins to be used as therapeutics. In addition, it is very expensive and a cheaper alternative needs to be developed. CHO-NEB3X media was the initial media tested for cells growth and consists of IMDM:Ham's F12 media (1:1) supplemented with lipids, vitamins, bovine serum albumin (BSA), insulin, transferrin, selenium and amino acids. Achieving a maximum viable cell density of 2×10^6 viable cells/ml in media that is free of animal derived products is the initial objective of these media development experiments. Currently, an experiment is being performed to optimize the amino acid, lipid, insulin, transferrin, and glutamine contents in the growth media. This experiment is set up with a central composite model design in order to limit the number of experiments, yet allow for interaction of components to be analyzed. Initial results have shown an improvement in both antibody productivity and cell density, although complete results are not yet available. In order to further optimize this media, samples from the spinner flasks will be analyzed for amino acid content, as well as glucose, glutamine and other components. While these experiments were not the focus of the project, they will be important for the long-term economical production of recombinant antibodies.

Conclusions: In conclusion, a significant amount of progress has been made towards generating cell lines that produce high levels of S25 antibody. In addition, the processes that we have developed to produce this antibody can be easily adapted for the quick production of future antibody candidates. An initial cell line, CHO-DG44 S25 #56, produced 2.3 μg/ml S25 antibody after 3 days of growth in adherent cultures. This same cell line produced 35 μg/ml S25 antibody after 6 days in suspension culture. Using dhfr mediated gene amplification, a cell line producing 48 μg/ml after 3 days in adherent culture was obtained. This was a great improvement over the original cell lines, although this cell line still needs to be transferred to suspension culture. Using as Protein A Sepharose Fast Flow resin (Pharmacia), we were able to produce mg quantities of a highly purified S25 antibody.

While there have been significant improvements in antibody production, there is still work to be done. The gene amplification procedure needs to be continued in order to isolate the highest producing clones. The next step is to clone the cell line CHO-DG44 S25 #3, which had the highest antibody productivity of any methotrexate resistant pool. In addition, the CHO-DG44 S25 #56 MTX7 clones are being transferred to increasing concentrations of methotrexate from 200 to 1000 nM, and screen these pools for S25 antibody production. In addition, the CHO-DG44 cells will be transfected with pS25-GFP in order to develop a protocol for cell screening based on GFP and flow cytometry. Lastly, long-term stability of gene copy number and protein production need to be confirmed by Southern blotting, Western blotting and ELISA.

Key Research Accomplishments

- Completed design and initiated construction of the shell space on third floor and basement of Othmer Hall.
- Generated CHO-DG44 cell line (CHO-DG44 S25 #56) with S25 antibody production (2.3 μg/ml after 3 days in culture)
- Transferred CHO-DG44 S25 #56 to suspension culture in CHO-S-SFM II media
- Cells grown in suspension culture for 6 days produced an S25 antibody concentration of 35 μg/ml
- Purified 23 mg of S25 antibody at 1 mg/ml in PBS
- Dhfr mediated gene amplification resulted in S25 antibody concentrations of 22 and 48 μg/ml in adherent culture, at methotrexate concentrations of 10 nM and 100 nM, respectively.
- Constucted a plasmid containing the S25 light and heavy chains, dhfr, and GFP-zeocin to be used in clonal selection via flow cytometry. (note: sequence and GFP/antibody production need to be confirmed).

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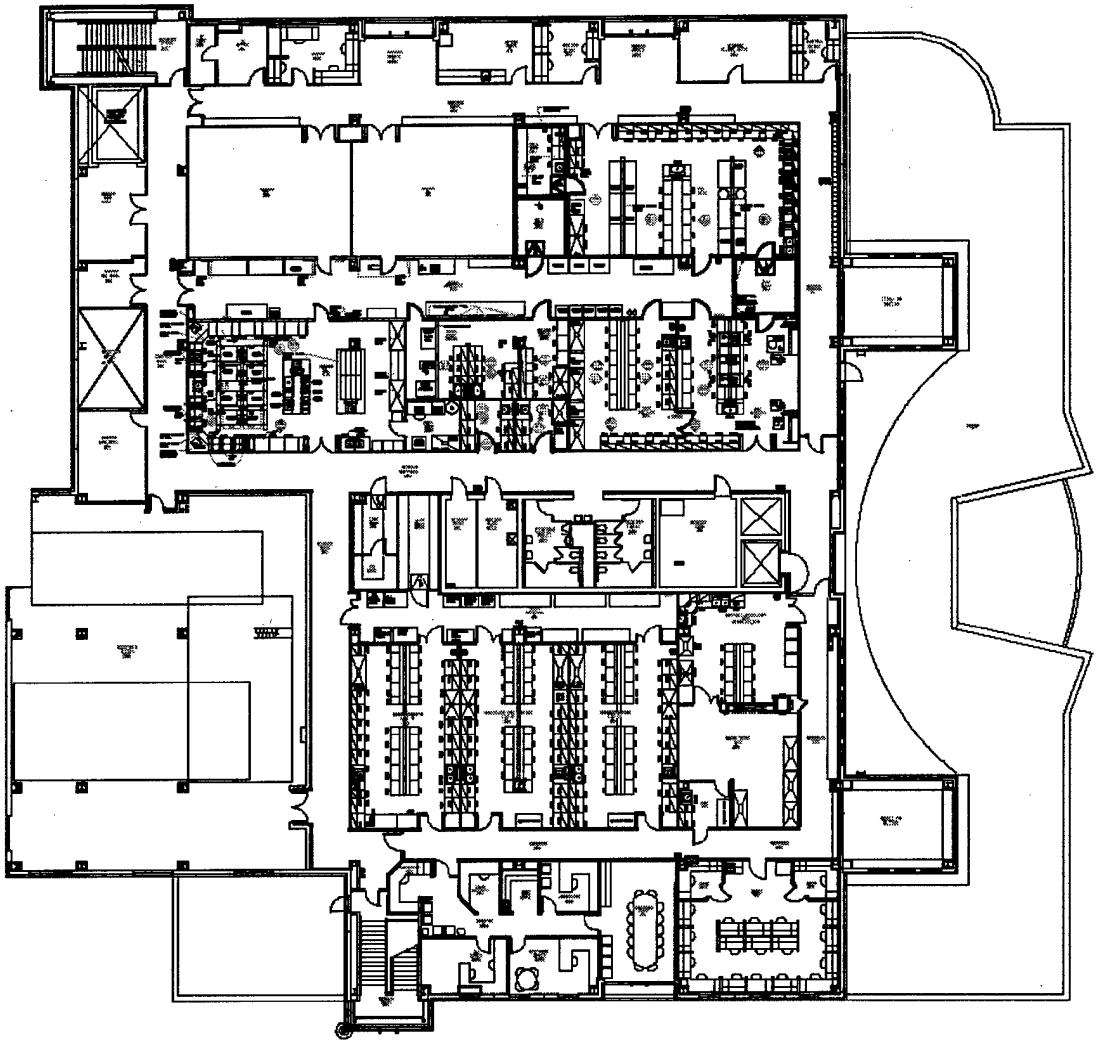
APPENDIX

Drawing of the third floor of Othmer Hall



Drawings of a section of the basement of Othmer Hall